

A natural pertactin deficient strain of *Bordetella pertussis* shows improved entry in human monocyte-derived dendritic cells

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SUMMARY

The invasion and the immunomodulatory effect of a *Bordetella pertussis* natural deficient strain 00141 (PRN⁻) on human dendritic cells (MDDC) and its *in vivo* infection ability in a mouse model were evaluated in comparison with the reference *B. pertussis* strain ATCC 97-97 (18323). The mutant was isolated from a case of pertussis which occurred in a 22-month-old infant with typical symptoms of the disease. The results showed that this natural *B. pertussis* PRN deficient strain presented higher invasion ability of human MDDC compared to the reference strain. This natural mutant similar to the *B. pertussis* reference strain had immunomodulatory properties, inducing maturation in the DC phenotype which resulted in the acquisition of potent T cell-activating properties and down-regulated IL-12 production, and secretion of IL-10. The ability of PRN⁻ strain to infect the lungs of CD1 mice was comparable to the reference strain and no difference was observed in the kinetics of clearance.

Overall, these results show that the enhanced ability of the PRN⁻ strain to invade/infect MDCC suggest that the PRN antigen may play a role in survival of the microorganism in the host.

KEY WORDS: Bordetella, Pertactin, Dendritic cells

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INTRODUCTION

Pertussis, or whooping cough, is a highly contagious disease of the tracheobronchial tree caused by *Bordetella pertussis*. Although the mechanism of *B. pertussis* pathogenesis is not fully understood, it is known that *B. pertussis* expresses factors such as filamentous hemagglutinin (FHA), pertussis toxin (PTX), and pertactin (PRN), which mediate adherence to upper respiratory epithelium cells, as a first step of infection. These proteins also seem to coordinate in adhesion of the bacteria to hu-

man epithelial phagocytic cells through various binding sites. In particular, in the sequence of the PRN protein, several motifs have been identified including a pair of tripeptide residues Arg-Gly-Asp (RGD) essential for cell binding. PRN has been considered an important antigen also in animal models and the presence of specific antibodies has been shown to correlate with clinical protection in humans (Charles *et al.*, 1991; Guiso *et al.*, 1999; Hellwig *et al.*, 2003; Roberts *et al.*, 1992; Roberts *et al.*, 1993). Furthermore, in clinical trials acellular vaccines containing PRN have been reported to provide better protection than other vaccines (Olin *et al.*, 1997).

In vitro studies of adhesion/invasion with different recipient cells showed that invasion depends on the *Bordetella* virulence gene (*bvg*) locus (Weingart and Weiss., 2000). To understand the role of the different adhesins in the infection process, *B. pertussis* strains each lacking one of

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the main adhesins, have been used, but conflicting results were obtained (Ishibashi *et al.*, 2001; van der Berg *et al.*, 1999).

Several studies have been carried out with PRN mutants to investigate their ability to adhere to and invade different mammalian cells, human laryngeal cell lines, Chinese Hamster Ovary cells and Hela cells (Leininger *et al.*, 1991). In most of these studies, the absence of this protein did not significantly influence the process except in the study described by Bassinet *et al.*, 2000 in which the presence of PRN conferred an inhibitory activity to the invasiveness of the human tracheal cell line (HTE). Besides interacting with epithelial cells of the respiratory tract, *B. pertussis* is recognized by cells of the innate immune system such as human monocytes (Stefanelli *et al.*, 2002; Shumilla *et al.*, 2004; Schaeffer and Weiss, 2001) and monocyte derived dendritic cells (MDDC) (McGuirk *et al.*, 2002; Tonon *et al.*, 2002; Fedele *et al.*, 2005; Spensieri *et al.*, 2006).

Increasing evidence suggests that DC are major players in innate immunity and trigger antimicrobial defense response through the production of inflammatory cytokines and other immune mediators, which in turn promote the development of specific T helper (Th) response that play a critical role in host protective immunity against infectious agents (Steinman RM. and Hammi H., 2006).

Here, we analyzed the behaviour of a natural PRN lacking *B. pertussis* strain, PRN⁻ 00414, isolated from an infant with whooping cough, in *in vitro* invasion, maturation and activities of the human MDDC. The murine respiratory infection model, previously described (Ausiello *et al.*, 2003; Guiso *et al.*, 1999) was also used to evaluate the PRN mutant behavior in both colonization and clearance from the mouse lungs. The experiments were performed in parallel with the *B. pertussis* strain ATCC 97-97 (18323) which is the recommended reference strain for this investigation.

MATERIALS AND METHODS

Bacterial strains: growth conditions and molecular characterization

B. pertussis ATCC reference strain 97-97 (18323) and *B. pertussis* PRN deficient mutant, PRN⁻ 00414, were grown at 37°C on charcoal agar plates supplemented with 10% of sheep blood (Oxoid)

for 48-72 hours. The mutant strain was previously characterized by Restriction Fragment Length Polymorphism (RFLP) of *Sma*I-digested DNA with a probe derived from the IS1002 and by DNA sequencing of the PT-S1 subunit (Mastrantonio *et al.*, 1999). The strain was also serotyped by anti-fimbriae monoclonal antibodies with a microagglutination test (Stefanelli *et al.*, 1999). Western-Blot analyses to detect the presence of PT, FHA and adenylate cyclase toxin (ACT) were performed following standard methods (Laemmli *et al.*, 1970) using specific polyclonal antibodies (polyclonal antibodies against PT and FHA were kindly provided by Dr. Mariagrazia Pizza, Novartis, Siena, Italy. Polyclonal antibodies against ACT were kindly provided by Dr. Nicole Guiso, Institute Pasteur, Paris, France).

Purification and culture of MDDC

Human monocytes were purified from peripheral blood of healthy donors as described elsewhere (Ausiello *et al.*, 2002). CD14⁺ cells were cultured at 5x10⁵/ml in RPMI 1640 (ICN-Flow, Aurora, OH) supplemented with heat-inactivated 10% LPS-screened fetal bovine serum (FBS, limulus amebocyte lysate <1 ng/ml) 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin (all from Hyclone Laboratories, Logan, UT) and 0.05 mM 2-mercaptoethanol (Sigma) (hereafter defined as complete medium), at 37°C in 5% CO₂, in the presence of 1000 U/ml hr IL-4 (Novartis Pharma AG, Basel, Switzerland) and 50 ng/ml hrGM-CSF (Novartis Pharma AG, Basel, Switzerland). After 6 days, immature (i) MDDC were washed and cytofluorometric analysis was performed for CD1a and CD14 expression.

MDCC infection and maturation

Bacteria, grown on charcoal agar plates, were resuspended within three hours before the experiment in 10 ml of prewarmed phosphate buffer saline (PBS) to a final concentration of 1x10⁹ CFU/ml. Serial dilutions were inoculated onto charcoal agar plates for accurate quantification of the multiplicity of infection (MOI) and bacteria were incubated with human immature MDDC (iMDDC), with a ratio of 100:1, for 2 hrs at 37°C in 5% CO₂. After a first wash with RPMI, plus polymixin (5 µg/ml) to remove extracellular bac-

teria, the MDDC were resuspended with RPMI with 10% of FCS plus polymixin (20 µg/ml), for 3 hrs at 37°C with 5% of CO₂, to kill any remaining extracellular bacteria. The infected MDDC were then lysed in cold distilled water, and intracellular bacteria were counted by plating appropriate dilutions onto charcoal agar plates and inspected up to 7 days of incubation.

The viability of intracellular bacteria was checked by counting them at different time points up to 24 h of incubation after infection. To make sure that adherent bacteria were not included in the count, parallel experiments with cytochalasin D, an inhibitor of phagocytosis, were performed.

To evaluate the induction of MDDC maturation and functions, infections were performed in the absence of polymixin B in order to avoid the killing of the bacterium. After 24 hours infected MDDC were harvested for immunophenotypic analysis and cytokine measurement by immunoenzymatic assay (ELISA).

Immunophenotypic analysis

MDDC were washed and re-suspended in PBS containing 3% FBS and 0.09% NaN₃, then incubated with a panel of fluorochrome-conjugated mAbs (obtained from BD Biosciences, San Jose, CA) specific for MDDC: anti-CD14, CD1a, CD80, CD83 and HLA-DR. Isotype-matched antibodies were used as negative control. Cells were analyzed with a FACScan (BD Biosciences). The *E. coli* LPS was used as control maturation stimulus. Fluorescence data are reported as % of positive cells when treatment induces the expression of the marker in cells that were negative; median fluorescence intensity (MFI) was used when treatment increased the expression of the marker in cells that were already positive.

Cytokine measurement by ELISA

To measure cytokine production, 0.5 ml of MDDC were cultured in the presence of the indicated stimuli in 5 ml tubes (Falcon, Becton Dickinson, Lincoln Park, NJ) at 37°C, 5% CO₂. Supernatants were collected after 24 hours, and IL-10 and IL-12 p70 production was assessed by ELISA (Quantikine, R&D Systems, Inc., Minneapolis, MN) with a sensitivity of 3.9 pg/ml for IL-10 and 5 pg/ml for IL-12. Optical density obtained was measured with a BioRad (Philadelphia, PA) 3550-UV Microplate Reader at 450 nm.

Intranasal infection of mice

Intranasal infection of mice was performed as previously described (Ausiello *et al.*, 2003). *B. pertussis* ATCC 97-97 (18323) and PRN⁻ 00414 were grown on charcoal agar plates for 48-72 hrs and the bacterial suspension for the inoculum of mice was prepared in physiological saline containing 1% casein. Briefly, CD1 mice were lightly ether anaesthetized and 50 µl containing 5x10⁶ viable bacteria were carefully instilled and allowed to be inhaled by the animal. For the determination of bacterial colonization of the lung tissues, groups of 4 mice were sacrificed after 2 h (time 0), and 2, 4, 7, 10 days after infection. The number of viable bacteria in the lungs, homogenized in 1.5 ml Casamino acid 3% (Oxoid), was determined by plating 10-fold serial dilutions on charcoal agar plates. The plates were inspected daily up to 7 days and the colony count was recorded. Data were normalized by transforming the mean of CFU for each time point for the total mice in log₁₀ CFU/lung.

Statistical analysis

The results were analyzed using the unpaired Student's *t* test. Differences, were considered significant at the P value of <0.05.

RESULTS

Molecular characteristics of *Bordetella pertussis* natural PRN deficient strain

The *B. pertussis* PRN⁻ 00414 strain showed an alteration in the region 2 of the *prn* gene due to the presence of the insertion sequence IS481 (Mastrantonio *et al.*, 1999) which is usually present in approximately 80 copies in the *B. pertussis* chromosome (MacLafferty *et al.*, 1988). This insertion made the strain unable to express the PRN protein *in vivo*. The PRN⁻ strain had fimbrial serotype 1,3, expressed the subunit A of the PT-S1, as detected also for the reference strain *B. pertussis* 18323 (data not shown), and belonged to RFLP type 29.

The presence of the other main antigens PTX, FHA and ACT detected by Western-Blot analysis using polyclonal antisera (Figure 1), confirmed the expression of *bvg*-activated gene products with a pattern identical to that of the control strain *B. pertussis* 18323.

Infection and *B. pertussis* survival in MDDC

Five hours after infection, time 0, as shown in Figure 2, the PRN⁻ strain had significantly increased invasion ability (50% of infected MDDC) compared to the wild reference strain 18323

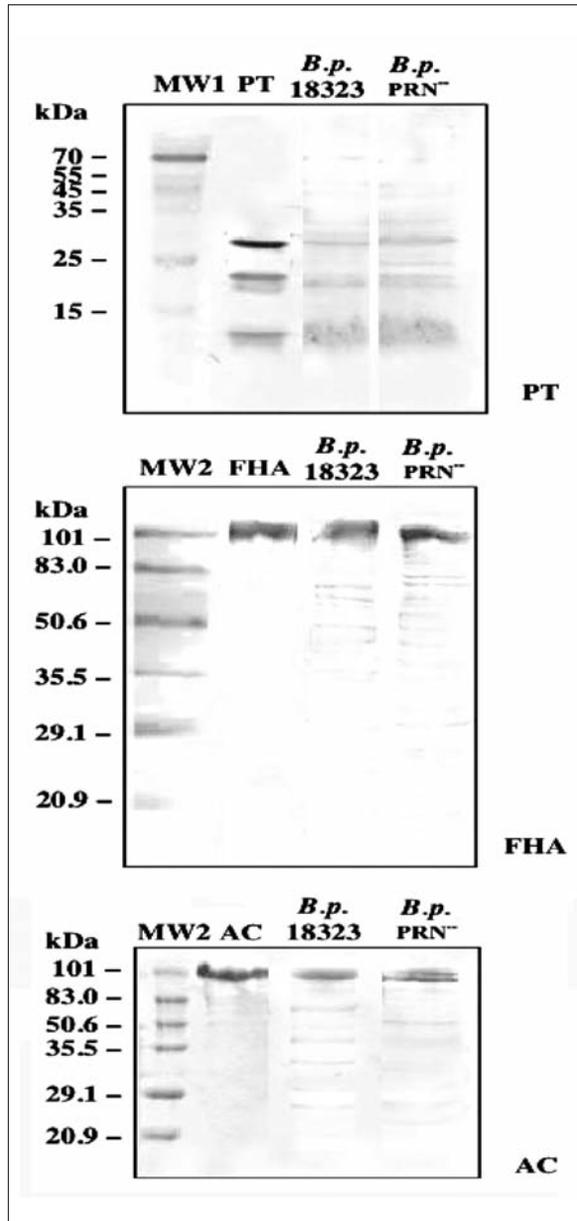


FIGURE 1 - Western-blot analysis of PT, FHA and ACT expression from the whole-cell lysates of *Bordetella pertussis* PRN⁻ 00414 and ATCC 18323 strains. MW1, PageRuler Prestained Protein Ladder (Fermentas LifeScience); MW2, Low Range Molecular weight (BioRad). PT, FHA and ACT purified control proteins were included in each panel.

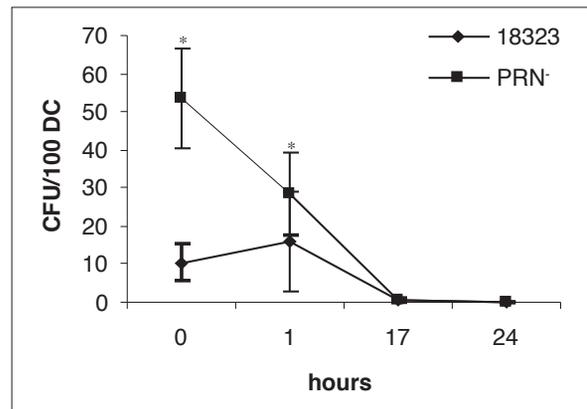


FIGURE 2 - Invasion of human monocyte derived dendritic cells (MDDC) by *Bordetella pertussis* ATCC 18323 (18323) and *Bordetella pertussis* PRN⁻ 00414 (PRN⁻) and kinetics of bacterial survival at different time points. Time 0 corresponds to 5 h after infection. The values represent the mean SE of three independent experiments.

which was able to infect around 10% of MDDC ($P < 0.05$) (Figure 2).

The bacterial survival in the MDDC showed a similar trend after the first 6 hours from the infection (time 1) for both strains, with a progressive fall in the number of viable bacteria at each observed time point (Figure 2). After 17 hours, no viable bacteria were recovered.

Phenotypic and cytokine analysis of bordetella-infected MDDC

As shown in Figure 3, after the infection of MDDC by each *B. pertussis* strain there was an increased expression of maturation markers such as the CD80, the CD83 and the HLA-DR molecules (Figure 3, panel A). The *B. pertussis* PRN⁻ mutant strain was able to promote the maturation process with the same efficiency observed for the reference strain and by the *E. coli* LPS. Both *Bordetella* strains induced high levels of IL-10 but not induction of IL-12, in MDDC. The control stimulus of the *E. coli* LPS was able to induce a great amount of IL-12 and IL-10 (Figure 3, panel B). The IL-10 amount induced by PRN⁻ strain was higher than that of *E. coli* LPS ($P < 0.05$) but comparable to that induced by *B. pertussis* 18323 strain.

Intranasal infection of mice

The intranasal infected mice were sacrificed at different times. The ability of the reference and mu-

FIGURE 3 - Panel A Influence of *Bordetella pertussis* ATCC 18323 (18323) and *Bordetella pertussis* PRN⁻ 00414 (PRN⁻) on human MDDC maturation. The expression of CD80 and HLA-DR was indicated by the median value of fluorescence (MFI), whereas the CD83 was indicated as percentage of expression. The analysis was performed 24 h after infection. The values represent the mean SE of three independent experiments. Panel B. Production of IL-10 and IL-12 by human MDDC after infection with *Bordetella pertussis* ATCC 18323 (18323) and *Bordetella pertussis* PRN⁻ 00414 (PRN⁻) strains. *E.coli* LPS was used as positive control (100ng/ml). The values represent the mean SE of three different experiments, * $P < 0.05$ vs LPS treated MDDC.

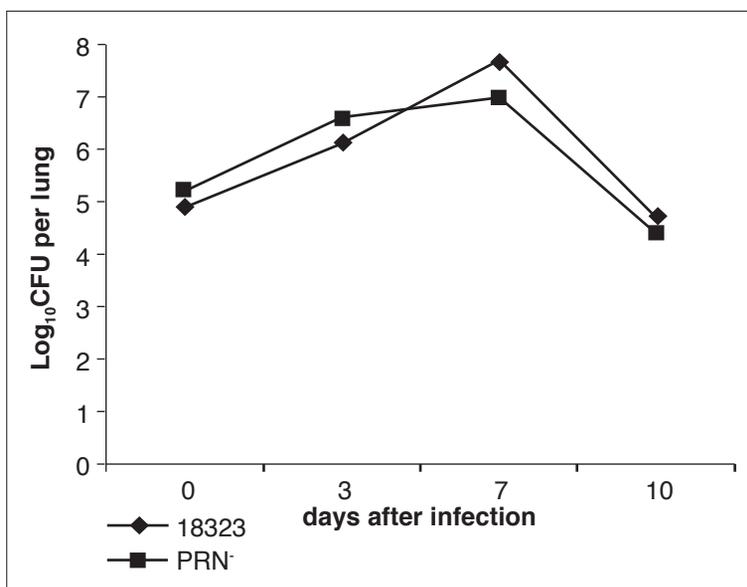
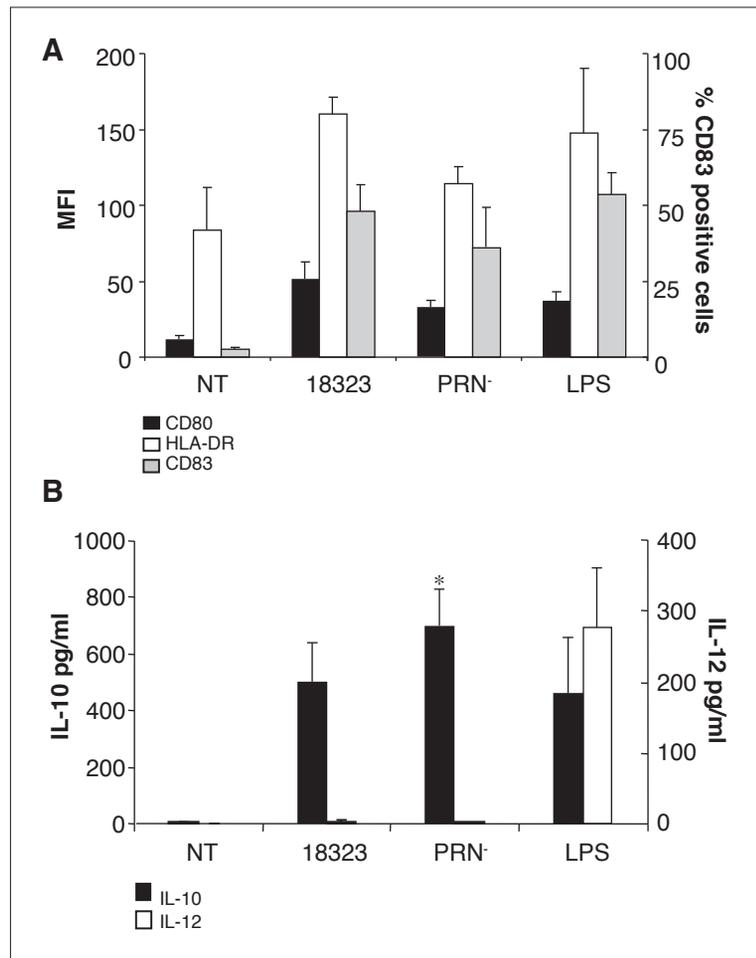


FIGURE 4 - Kinetics of clearance from lungs at different time points of *Bordetella pertussis* ATCC 18323 (18323) and *Bordetella pertussis* PRN⁻ 00414 (PRN⁻) strain, after intranasal infection of CD1 mice. The results are expressed as the mean of three experiments.

tant strains to colonize and proliferate in the respiratory tract of the mice is expressed as the result of the number of CFUs recovered from the lungs. All the animals had between 10^4 and 10^5 CFU/lung of each strain at the beginning of the infection. After the first day, both strains started actively to multiply and they were detected for at least 10 days after infection. The CFU counts were comparable for both strains and no difference in the kinetics of bacterial clearance was observed (Figure 4).

CONCLUSIONS

This study evaluated the infection and immunomodulatory effect on human MDDC of a *B. pertussis* natural PRN⁻ strain as well as the *in vivo* infection ability in a mouse model, in comparison with the *B. pertussis* reference ATCC 97-97 (18323) strain.

The mutant was isolated from a 22-month-old infant presenting the typical symptoms of whooping cough. Only limited data are currently available on phagocytosis and survival of *B. pertussis* in human MDDC (Fedele, *et al.*, 2005). Due to pivotal role of DC in bridging natural and specific immune response, these studies are valuable to understand the T cell responses during whooping cough or after administration of pertussis vaccines. Indeed, protection against *B. pertussis* was found to depend on both antibodies for early bacterial clearance and on Th1-type responses for the long lasting immunity conferred by previous infection or immunization with whole-cell pertussis vaccines (Godfroid *et al.*, 2004; Cassone *et al.*, 1997; Ausiello *et al.*, 1997; Mills, 2001; Ryan *et al.*, 1998; Ausiello *et al.*, 1999).

Several studies have been performed to evaluate the phagocytosis of *B. pertussis in vitro* mutant strains by different cell lines (Roberts *et al.*, 1991; Van den Berg *et al.*, 1999). However, the results were often contradictory (Bassinnet *et al.*, 2000; Van den Berg *et al.*, 1999). In particular, the PRN protein has been considered an important immunogen, but the exact role of the protein in the pathogenesis is still unclear. Different hypotheses have been suggested such as its possible interaction with one of the major pertussis toxins, i.e. the adenylate cyclase, especially during the phase I of the infection and with the major adhesin FHA (Bassinnet *et al.*, 2000).

The results of this study show that a *B. pertussis* strain, lacking the PRN protein, can infect human MDDC and the rate of phagocytosis is higher than that of the *B. pertussis* control strain and, in particular, more than 50 % ($P < 0.05$) of the cells resulted infected compared to 10%, respectively. A similar result for an *in vitro* PRN⁻ mutant was observed using a human tracheal epithelial cell line HTE (Bassinnet *et al.*, 2000).

Several reports suggested that *B. pertussis* is able to survive inside human monocytes and macrophages but not in human neutrophils (Lenz *et al.*, 2000; Weingart and Weiss, 2000). However, it has been hypothesized that intracellular survival is only transient and that *B. pertussis* kills monocytes and macrophages (Khelef and Guiso, 1995). In this study, the survival in human MDDC of both mutant and control strains was similar suggesting that other antigens may be involved in the resistance to intracellular killing. In addition, the PRN⁻ mutant was able to induce MDDC maturation like the control strain indicating that this process is influenced in a multifactor manner. Different studies have been conducted by using the intranasal *B. pertussis* infection in mice, including those analyzing strains deficient for different virulence factors. In particular, FHA and fimbriae negative mutants showed a decreased ability to colonize the trachea but kept a similar ability to colonize the lungs of mice (Geuijen *et al.*, 1997; Vandebriel *et al.*, 2003). Also in our study the results showed that mutant and control strains had a comparable ability to infect the mouse lungs.

Overall, the results of this study show that a natural *B. pertussis* PRN deficient strain had immunomodulatory properties on human MDDC similar to those of the 18323 reference strain. Both strains induced changes in the MDDC phenotype which resulted in acquisition of potent T cell-activating properties via IL-10 production. The most important result of this natural defective mutant is linked to its enhanced ability to invade MDDC suggesting that PRN protein prevents bacterial adherence and/or internalization. It can be hypothesized that by masking this antigen anti-PRN antibodies induced by vaccination could improve phagocytosis by DC, contributing to the elimination of Bordetellae. This mechanism might justify the higher efficacy of the three-component pertussis acellular vaccines compared

to those lacking the PRN antigen, as suggested in a placebo-controlled pertussis vaccine trial conducted in the past (Olin *et al.*, 1997) and in the mouse model (Mills *et al.*, 1998).

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